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Generation of Interleukin-8 from Human Monocytes in Response to *Trichomonas vaginalis* Stimulation

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Neutrophils are the predominant inflammatory cells found in the vaginal discharges of patients with *Trichomonas vaginalis* infection. We have investigated the possible role of interleukin-8 (IL-8) in the inflammatory response elicited by *T. vaginalis* infection. This study has shown that *T. vaginalis* induces blood monocytes to produce large amounts of bioactive IL-8, mainly by membrane components of *T. vaginalis* (MTV). Monocyte-derived IL-8 induced by MTV was dose and time dependent. The peak level of IL-8 was 102 ± 11 ng/ml of conditioned media (mean ± standard error; $n = 5$) obtained from MTV-stimulated monocytes (MTVCM) at 36 h of cultivation. With a multichamber chemotactic assay, we found an optimal neutrophil chemotaxis (177 ± 14 migrated cells) induced by MTVCM collected at 16 h of cultivation when the level of IL-8 was 42 ± 8 ng/ml. A neutralizing monoclonal antibody directed against IL-8, but not the irrelevant antibodies, significantly blocked the neutrophil chemotactic activity (decreased from 153 ± 6 to 23 ± 3 migrated cells; $n = 3$ [$P < 0.001$]) induced by MTVCM. Moreover, the maximum increase of the IL-8 mRNA level from MTV-treated monocytes was observed after a 5-h cultivation and decreased thereafter. Monocytes cocultured with MTV in the presence of a neutralizing monoclonal antibody directed against tumor necrosis factor alpha, but not against IL-1β, decreased IL-8 production by 25% ($P < 0.05$), indicating that the release of IL-8 in MTV-stimulated monocytes is partially dependent on tumor necrosis factor alpha. The capacity of MTV-induced monocytes to synthesize IL-8 suggests that these cells can contribute to the induction of the acute inflammatory response seen in *T. vaginalis* infection.

Trichomoniasis is one of the major sexually transmitted disease in humans. A prominent cytological change observed in trichomoniasis is the infiltration of leukocytes, particularly neutrophils (16). Although the mechanisms of leukocyte infiltration are not fully understood, it is generally believed that an important role is played by chemoattractants generated at the reaction sites. Of the known chemoattractants involved in the inflammatory response of *Trichomonas vaginalis* infection, complement components have been documented to play a role in the activation of neutrophils against this organism (27, 30). Since *T. vaginalis* interacts with leukocytes in the inflammatory response, it is rationally presumed that leukocyte-derived chemoattractants may be generated during *T. vaginalis* infection. We have found high levels of interleukin-8 (IL-8) and leukotriene B_4 (LTB_4) in vaginal discharges from patients with symptomatic trichomoniasis (31, 34). The presence of LTB_4 in vaginal discharges from patients with trichomoniasis could be due to the spontaneous release of LTB_4 from *T. vaginalis* (33) and/or from neutrophils induced by the interaction of trichomonads and humoral immunity (32). Recent studies have indicated that IL-8, a novel chemotactic and activating cytokine with high selectivity for neutrophils (5), enhances the antimicrobial activities of neutrophils by inducing neutrophil degranulation, respiratory burst, and 5-lipoxygenase activity (14, 23, 26, 28). In this study, the cellular and molecular mechanism for monocyte-derived IL-8 production by *T. vaginalis* was elucidated. Results from this study could provide a more comprehensive understanding of the immunopathogenesis of *T. vaginalis* infection.

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MATERIALS AND METHODS

Organisms. Several local isolates of *T. vaginalis*, axenically cultivated, were maintained in a modified medium identical to the TYI-S-33 medium of Diamond et al. (13), except that 0.5% Panmede (Paines & Byrne Limited, Greenford, England) was added. The number of organisms per culture was determined with a Coulter Counter (model D industrial; Coulter Electronics, Inc., Hialeah, Fla.) with a 70-μm-aperture tube.

Preparation of trichomonad components. (i) *T. vaginalis* secretory components (STV). Trichomonads harvested at the logarithmic phase were washed twice with phosphate-buffered saline (PBS; pH 7.3), resuspended in Hanks' balanced salt solution at 10^7 organisms per ml, and incubated at 37°C for 1 h. Parasites were sedimented by light centrifugation, and the supernatant (STV) was collected and filter sterilized (0.22-μm pore size; Minisart [Sartorius, Goettingen, Germany]). After concentration by cryocentrifugation and dialysis against PBS, the protein concentration was quantitated and adjusted to 5 mg/ml by the Bradford protein assay (Bio-Rad, Hercules, Calif.).

(ii) *T. vaginalis* membrane components (MTV). The membrane fraction of trichomonads was extracted by a modification of a method described previously (3). Briefly, the sedimented organisms from the preparation described above were homogenized in Tris-lysing buffer containing 1 mM EDTA (Sigma Chemical Co., St. Louis, Mo.), 1 mM EGTA [ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Sigma], and 1 mM phenylmethylsulfonyl fluoride (Sigma) by 20 strokes of a glass Dounce homogenizer and centrifuged at 1,000 × *g* for 10 min. Supernatants were collected and centrifuged at 50,000 × *g* for 30 min with an 80 Ti rotor in an ultracentrifuge (model L8-80M; Beckman Instruments, Inc., Fullerton, Calif.). Pellets were collected and resuspended in lysing buffer containing 1.5% *n*-octyl glucoside (Sigma), sonicated at 20 kHz with three 15-s intervals, and then centrifuged at 10,000 × *g* for 30 min. Supernatants were collected, dialyzed against PBS, and filter sterilized. The protein concentration was adjusted to 5 mg/ml.

(iii) *T. vaginalis* whole parasites (WTV). The whole trichomonads were prepared by three washes of *T. vaginalis* with PBS and resuspended in serum-free macrophage culture medium (Life Technologies, Gibco-BRL, Gaithersburg, Md.) at 10^7 trichomonads per ml.

Preparation of monocytes and neutrophils. Venous blood from healthy donors was drawn into plastic syringes containing 10 IU of heparin per ml. Dextran (4.5% [wt/vol]; T500 [Pharmacia, Uppsala, Sweden]) was added at a ratio of 1:5, and the syringe was incubated nozzle upward at 37°C for 30 min. The upper fraction of leukocyte-enriched plasma was then layered onto Ficoll-Paque (Pharmacia) at a ratio of 2:1 and centrifuged at 1,500 × *g* for 10 min. The separation of monocytes from the mononuclear leukocytes (10^7 cells per ml) at the interface of plasma and Ficoll-Paque was performed by discontinuous Percoll density

gradient centrifugation (4). Monocytes were washed twice with RPMI 1640 medium (Gibco-BRL). Finally, the monocyte suspension was adjusted to 10^6 cells per ml of serum-free macrophage culture medium (Gibco-BRL). Cells were >95% viable, as determined by trypan blue exclusion, and consisted of >90% monocytes, as assessed by Giemsa stain and nonspecific esterase staining. Pellets at the bottom of the centrifuge tubes were washed, and residual erythrocytes were lysed by distilled-water treatment for 15 s. This hypotonic shock was stopped by adding an equal volume of 1.8% sodium chloride solution. Cells were finally suspended to 10^7 cells per ml in Hanks' balanced salt solution. By this method, the purity and viability of neutrophils consistently exceeded 95 and 98%, respectively.

Preparation of conditioned media. Cultivation of monocytes (10^6/ml) with various concentrations of STV, MTV, and WTV was carried out in Teflon containers (5 by 5 cm; Savillex, Minneapolis, Minn.). At different time intervals, culture supernatants were collected, filter sterilized, and aliquoted. The supernatants harvested from STV-, MTV-, and WTV-stimulated cultures were referred as STVCM, MTVCM, and WTVCM, respectively. Membrane fractions from *Pentatrichomonas hominis* (ATCC 30098) and human mononuclear leukocytes were prepared as described in the previous section and included as controls. Conditioned medium from cell culture without stimulation (CCM) was used as a negative control. In some experiments, monoclonal antibodies directed against tumor necrosis factor alpha (TNF-α) and IL-1β were added to monocyte cultures to determine their effects on IL-8 production. Aliquots of STV, MTV, and WTV were checked for endotoxin contamination by *Limulus* amebocyte lysate assay (Pyrogent Plus; Bio-Whittaker, Walkersville, Md.). Both lipopolysaccharides (LPS; *Escherichia coli* O111:B4 [Sigma]) and polymyxin B (10 μg/ml; Sigma) were used for examination of the endotoxin effect. All of the procedures described above were carried out in sterile conditions and with reagents prepared with endotoxin-free water for clinical use.

Chemotactic assay. Chemotaxis was assessed in a 48-well microchemotaxis chamber (Neuro Probe, Inc., Cabin John, Md.) by a method described previously (33). Various concentrations of STVCM, MTVCM, WTVCM, and CCM were tested for the chemotactic activity by neutrophils. Serum-free macrophage culture medium was used as a negative control. Recombinant human IL-8 (rhIL-8, 10 nM; R&D Systems, Minneapolis, Minn.), *N*-formylmethionylleucyl phenylalanine (FMLP, 10 nM; Sigma), and LTB_4 (10 nM; Amersham, Buckinghamshire, England) were used as positive controls. In some experiments, conditioned media were heated at 56°C for 60 min or deproteinized by enzymatic digestion with 50 μg of proteinase K (Boehringer GmbH, Mannheim, Germany) per ml at 56°C for 60 min. Pretreatments of conditioned media with a neutralizing monoclonal anti-human IL-8 antibody (mouse immunoglobulin G1 [IgG1]; R&D Systems) or irrelevant control monoclonal antibodies (anti-human IL-1β, anti-human TNF-α, and anti-human granulocyte-macrophage colony-stimulating factor [GM-CSF], all mouse IgG1s; R&D Systems) were carried out at 4°C for 30 min. Migrated cells were counted in five random high-power fields (magnification, ×400) per well, and the results were expressed as the mean number of cells in five high-power fields per triplicate well.

ELISA for cytokines. Levels of IL-8, IL-1β, and TNF-α in STVCM, MTVCM, WTVCM, and CCM were determined with commercialized enzyme-linked immunosorbent assay (ELISA) kits (Quantikine; R&D Systems). This sandwich ELISA recognized only the individual cytokine tested and exhibited no detectable cross-reactivity to other cytokines tested, according to the manufacturer's description. Sensitivities of ELISA for IL-8, IL-1β, and TNF-α were 30, 0.3, and 4.4 pg/ml, respectively. For the determination of IL-8 in culture media, for example, a murine monoclonal antibody directed against IL-8 was used as the capturing antibody, while horseradish peroxidase-conjugated polyclonal antibody directed against IL-8 was used as the detecting antibody. A colorimetric reaction was started by adding a mixture of hydrogen peroxide and chromogen (tetramethyl benzidine). The reaction was terminated by adding 2 N sulfuric acid. Results were read at 450 nm with a correction wavelength at 570 nm.

RNA preparation and Northern (RNA) blot analysis. Total cellular RNA from monocytes (5×10^6) was extracted by use of acid guanidinium thiocyanate-phenol-chloroform as described previously (9). Twenty micrograms of total RNA was size fractionated by Northern blot analysis with formaldehyde–1% agarose gels, transferred onto nitrocellulose filters, baked, prehybridized, and hybridized to one of the following digoxigenin (digoxigenin-dUTP; Boehringer)-labeled cDNA probes: (i) the 0.6-kb *Eco*RI insert of human IL-8 cDNA purified from plasmid pUC19 (21) or (ii) the 1.25-kb *Pst*I insert of human glyceraldehyde-3-phosphate dehydrogenase cDNA purified from plasmid pIBI30 (39). Hybridization was performed at 42°C for 16 h in a solution containing 50% (vol/vol) formamide, 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate), 1× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 200 μg of salmon sperm DNA per ml (37). Filters were washed three times in 2× SSC–0.1% SDS at room temperature for 30 min and twice in 0.1× SSC–0.1% SDS at 68°C for 15 min. After blocking, filters were treated with a 1:10,000 dilution of anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer) at room temperature for 30 min and then washed three times for 15 min each time. The substrate Lumigen PPD [4-methoxy-4-(3-phosphatephenyl)spiro-(1,2-dioxetane-3,2′-adamantane); Boehringer GmbH], in the presence of basic buffer, was used to react with alkaline phosphatase and generate light via an unstable intermediate, which can be documented on X-ray film. The damp filters were sealed in a hybridization bag, and the sealed filters were preincubated for 15 min at 37°C. The sealed filters were then exposed to X-ray film for 30 min at room temperature. Quantitation of the chemiluminescence exposure was performed with a Phosphorimager (Molecular Dynamics, Sunnyvale, Calif.).

mRNA stability analysis. The rate of IL-8 mRNA decay from LPS- and MTV-stimulated monocytes was determined by mRNA decay analysis. Monocytes were treated with LPS (10 μg/ml) or MTV (50 μg/ml), RNA synthesis was blocked by adding actinomycin D (Sigma) after 2 h in culture, and then total cellular RNA was isolated at 0.5, 1, 2, 3, and 4 h after the addition of actinomycin D (10 μg/ml). Duplicate stimulated cell samples were tested in the absence of actinomycin D. Northern blot analysis was then performed, and the decay of mRNA was determined with a Molecular Dynamics Phosphorimager.

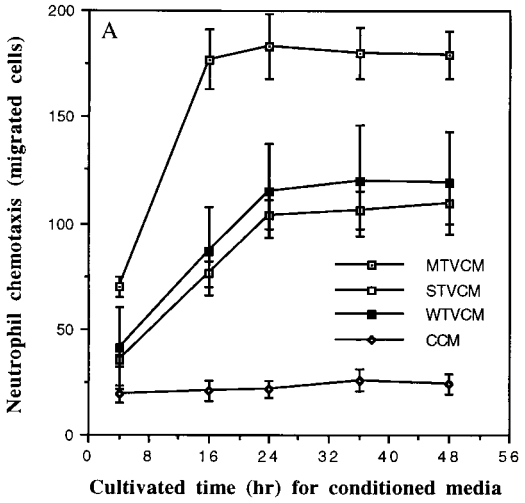
Statistical analysis. Data are expressed as means ± standard errors (SEs). The statistical evaluation of the data was performed with Student's *t* test for paired data and considered significant if P was <0.05.

RESULTS

Effect of *T. vaginalis* components on the production of neutrophil chemoattractants by monocytes. Human blood monocytes (10^6/ml) were incubated with a variety of trichomonad components (STV [50 μg/ml], MTV [50 μg/ml], and WTV [10^6 trichomonads per ml]), and the respective conditioned media (STVCM, MTVCM, and WTVCM) were collected at different time intervals. The dosages used for stimulation of monocytes were optimized to produce a maximum effect as determined by neutrophil chemotaxis (data not shown). Figure 1A clearly shows that both STVCM and WTVCM induce neutrophil chemotaxis in similar patterns, at levels which appear significantly lower than that induced by MTVCM ($P < 0.01$). A significant increase of IL-8 in MTVCM was noted at 4 h of cultivation (12 ± 3 ng/ml, $n = 5$) and progressively reached the maximum level by 36 h (102 ± 11 ng/ml) (Fig. 1B). After a 16-h cultivation, the level of IL-8 in MTVCM was 42 ± 8 ng/ml, and the maximum neutrophil chemotaxis (177 ± 14 migrated cells, $n = 5$) was observed (Fig. 1A). With rhIL-8 as a standard control, at the range of 1 to 40 ng/ml, IL-8 induced neutrophil chemotaxis in a dose-dependent manner, while at a level over 40 ng/ml, neutrophil chemotaxis reached a plateau (Fig. 2A). For STVCM and WTVCM, the maximum amount of IL-8 detected was in the range of 20 to 30 ng/ml throughout the period of cultivation. IL-8 in CCM was consistently at a low level (<3 ng/ml) during the period of monocyte culture (Fig. 1B).

Neutrophil chemotactic activity in MTV. Because MTVCM is largely responsible for neutrophil chemotaxis, MTVCM has been used for further investigation throughout this study. Our preliminary study revealed that each batch of MTV preparation (from seven isolates) itself did not affect leukocyte viability and motility. At the protein level of 50 μg/ml, MTV had an optimal effect on the production of IL-8 by monocytes (Fig. 2B). The neutrophil chemotaxis induced by MTVCM was in parallel with the production of IL-8 by monocytes stimulated with MTV. When the level of IL-8 in MTVCM was in the range of 10 to 40 ng/ml, MTVCM induced neutrophil chemotaxis in a dose-dependent manner. Checkerboard analysis was performed to determine whether the increased migration of neutrophils in response to MTVCM was due to increased random migration (chemokinesis) or chemotaxis. The results suggest that MTVCM enhances neutrophil migration by chemotaxis rather than chemokinesis (Table 1). Heat treatment of MTVCM (56°C, 60 min) did not diminish the neutrophil chemotaxis, but proteinase K treatment (50 μg/ml, 56°C, 60 min) eliminated the neutrophil chemotaxis induced by MTVCM (Table 2).

To exclude the possibility of simple release of the preformed IL-8 from human monocytes by damaging monocytes with MTV, cell viability was assessed by trypan blue exclusion at different time intervals. Viable cells in a range of 90 to 95% were observed throughout the time of cultivation. The membrane fraction of human mononuclear leukocytes did not in-



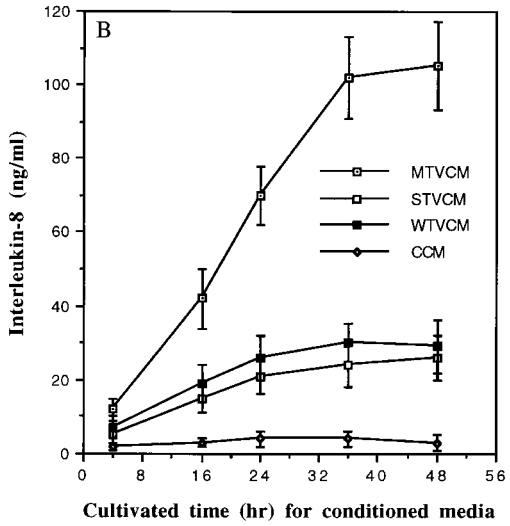
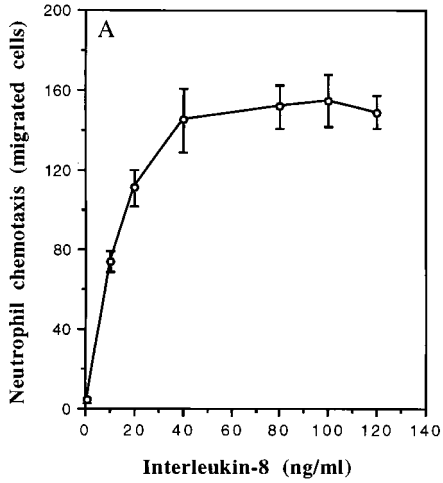


FIG. 1. Effect of trichomonad components on the production of neutrophil chemotactic activity (A) and IL-8 (B) by monocytes. A significant increase of IL-8 in MTVCM was found at 4 h of cultivation (12 ± 3 ng/ml) and reached a plateau by 36 h (102 ± 11 ng/ml). After a 16-h cultivation, the level of IL-8 in MTVCM was 42 ± 8 ng/ml, and maximum neutrophil chemotaxis (177 ± 14 migrated cells, $n = 5$) was observed. For STVCM and WTVCM, the maximum amount of IL-8 detected was in the range of 20 to 30 ng/ml throughout the period of cultivation.



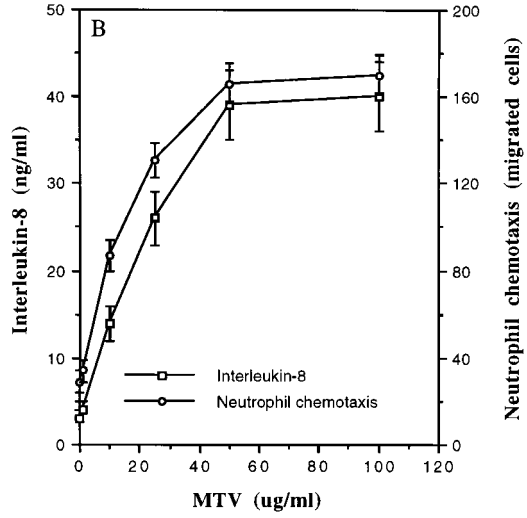


FIG. 2. (A) Induction of neutrophil chemotaxis by IL-8. With rhIL-8 as a standard control, in the range of 1 to 40 ng/ml, IL-8 induced neutrophil chemotaxis in a dose-dependent manner; at a level over 40 ng/ml, IL-8 induced neutrophil chemotaxis to its maximum. (B) Dose effect of MTV on the production of IL-8 by monocytes. Maximum production of IL-8 was observed when monocytes were stimulated with MTV at a protein level of 50 μg/ml after a 16-h cultivation.

duce IL-8 production by monocytes, while that of both *T. vaginalis* and *P. hominis* stimulated monocytes to produce a large amount of bioactive IL-8 (Fig. 3A). This suggests that membrane components inducing IL-8 production from monocytes are of parasite rather than host origin.

The levels of endotoxins in each batch of membrane components and culture medium were less than 10 pg/ml, as checked by *Limulus* assay. Moreover, we found that polymyxin B, at 10 μg/ml, completely inhibited the generation of IL-8 from monocytes by LPS at 100 ng/ml (Fig. 3A). Neither the IL-8 level nor the neutrophil chemotactic activity in MTVCM is affected by the addition of polymyxin B to the monocyte cultures (Fig. 3). Therefore, it appears unlikely that the minute amount of endotoxin contamination in the MTV preparation may contribute to IL-8 production by monocytes in this study.

Neither IL-1β nor TNF-α was detected in supernatants from controlled monocyte cultures. However, TNF-α, but not IL-1β, could be detected in a range of 50 to 100 pg/ml throughout the period of monocyte culture with MTV (data not shown). Cultivation of monocytes with MTV in the presence of a neutralizing monoclonal anti-TNF-α antibody (5 μg/ml), but not anti-IL-1β, decreased IL-8 production by 25% ($P < 0.05$) (Table 3). The effect of TNF-α on IL-8 production by monocytes was therefore determined. The addition of a minute amount of TNF-α (100 pg/ml) to monocyte cultures slightly enhanced IL-8 production compared with that of nontreated controls (3.2 ± 0.3 versus 2.2 ± 0.2 ng/ml, $n = 3$, $P < 0.05$). The

TABLE 1. Checkboard analysis of neutrophil chemotaxis by MTVCM

MTVCM concn (%) in bottom chamber	No. of neutrophils that migrated through filters at MTVCM concn in top chamber of[a]:				
	0%[b]	10%	25%	50%	100%
0	18 ± 7*	23 ± 5	25 ± 7	24 ± 5	26 ± 3
10	25 ± 6**	27 ± 8			
25	41 ± 7†		28 ± 8		
50	95 ± 6‡			27 ± 5	
100	156 ± 5§				29 ± 4

[a] The MTVCM used was taken at 16 h of cultivation. Data represent means ± SEs of three separate experiments performed in triplicate.
[b] * versus **, not significant; ** versus †, $P < 0.05$; † versus ‡, $P < 0.001$; ‡ versus §, $P < 0.001$.

increase of TNF-α from 1 to 10 ng/ml in monocyte cultures was proportional to the augmentation of IL-8 production (from 6.2 ± 0.5 to 13.8 ± 0.6 ng/ml). Moreover, a large amount of exogenous TNF-α (20 ng/ml) added to monocyte cultures with MTV markedly increased IL-8 production (Table 3). Nevertheless, monoclonal antibody directed against TNF-α (5 μg/ml) completely blocked the IL-8 response of monocytes to TNF-α (20 ng/ml) added to the cultures (Table 3).

Effect of anti-IL-8 monoclonal antibodies on MTVCM-induced neutrophil chemotaxis. To determine whether IL-8 in MTVCM is the factor responsible for neutrophil chemotaxis, we used a panel of monoclonal antibodies, directed against IL-8, GM-CSF, IL-1β, and TNF-α, respectively, to evaluate their neutralizing effects on neutrophil chemotaxis induced by MTVCM (Table 4). A monoclonal antibody directed against IL-8 (IgG1) at the level of 5 μg/ml almost completely abolished the neutrophil chemotaxis induced by MTVCM, while other monoclonal antibodies (directed against GM-CSF, IL-1β, or TNF-α [all IgG1]) did not work. Even with rhIL-8 used as a positive control (80 ng/ml [10 nM]), neutrophil chemotaxis was entirely reversed by monoclonal anti-IL-8 antibody treatment. Furthermore, monoclonal antibody directed against IL-8 did not affect neutrophil chemotaxis induced by LTB_4 (10 nM) or FMLP (10 nM). This indicates that IL-8 is the responsible factor contributing to neutrophil chemotaxis induced by MTVCM.

IL-8 gene expression by human monocytes stimulated with MTV. Monocytes showed low levels of IL-8 gene expression in the control state, but after a 3-h cultivation with MTV (50 μg/ml), monocytes showed a marked increase in the levels of IL-8 gene expression (Fig. 4A). IL-8 gene expression peaked at 5 h of cultivation with MTV and decreased thereafter. In contrast, LPS-induced monocyte IL-8 mRNA expression was persistently high at 24 h of cultivation (Fig. 4B). The induction of IL-8 mRNA was paralleled by a dose-dependent increase in the expression of IL-8 protein (data not shown). To assess whether the induction of IL-8 mRNA by MTV was dependent on de novo protein synthesis, MTV-treated monocytes were incubated in the presence or absence of the protein synthesis inhibitor cycloheximide (5 μg/ml; Sigma), and total RNA was extracted after a 4-h cultivation. As shown in Fig. 5, treatment of monocytes with cycloheximide alone resulted in modest superinduction of IL-8 mRNA expression. Treatment of monocytes with MTV resulted in upregulation of IL-8 mRNA compared with that of the control, with substantial superinduction (50% increase) of IL-8 mRNA observed after concomitant treatment with cycloheximide. On the other hand, in the case of LPS-stimulated monocytes, no superinduction of IL-8 mRNA expression could be found (Fig. 5). To assess whether differences in the regulation of IL-8 mRNA induced by MTV and LPS were attributable to differences in mRNA stability, we performed mRNA stability studies. The half-life of IL-8

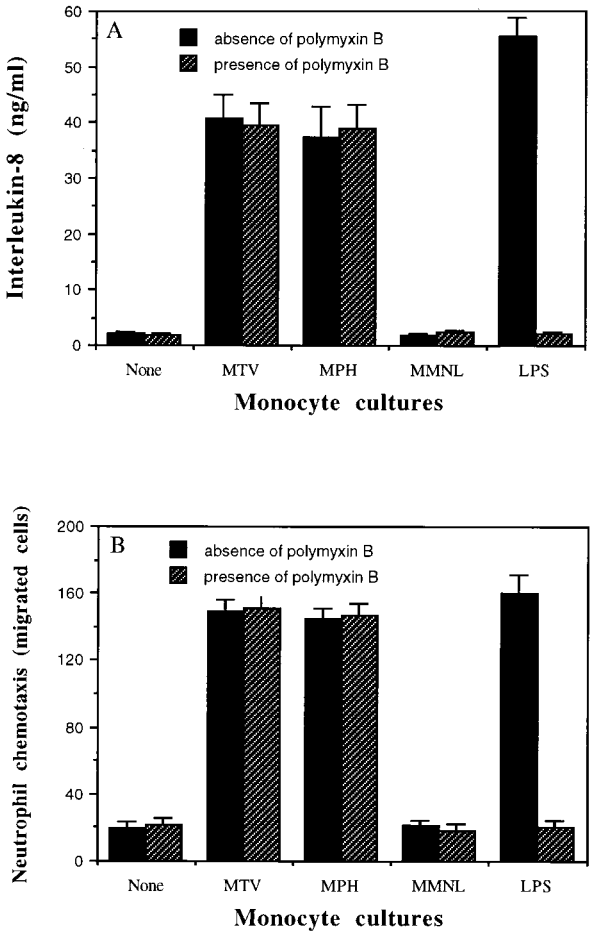


FIG. 3. IL-8 production by monocytes (A) and their induction of neutrophil chemotaxis (B) in the presence of various stimuli. MTV (50 μg/ml) and membrane components from *P. hominis* (MPH; 50 μg/ml) and mononuclear leukocytes (MMNL; 50 μg/ml) themselves were used as controls and had no chemotactic activities. The concentrations of LPS and polymyxin B used were 100 ng/ml and 10 μg/ml, respectively. Neither the IL-8 level nor the neutrophil chemotactic activity in MTV and *P. hominis* membrane component-conditioned media was affected by the addition of polymyxin B to the monocyte cultures, while both in LPS-conditioned medium were almost completely inhibited by the presence of polymyxin B.

TABLE 2. Effect of heat and proteinase K on neutrophil chemotaxis by MTVCM

Chemoattractant[a]	Neutrophil chemotaxis (no. of cells that migrated) induced by MTVCM pretreated with[b]:		
	Nothing	Heat[c]	Proteinase K[d]
CCM	35 ± 6	41 ± 8	0
MTVCM	169 ± 5	179 ± 7	0
IL-8 (10 nM)	188 ± 10	174 ± 16	0
LTB_4 (10 nM)	121 ± 14	118 ± 8	114 ± 9

[a] The CCM and MTVCM used were taken at 16 h of cultivation.
[b] Data represent means ± SEs of two separate experiments performed in duplicate.
[c] MTVCM was pretreated by heating at 56°C for 60 min.
[d] MTVCM was pretreated with proteinase K (50 μg/ml) at 56°C for 60 min.

TABLE 3. Effect of TNF-α on IL-8 generation from monocytes stimulated by MTV[a]

Monocytes stimulated with	IL-8 produced (ng/ml)[b]
None	1.9 ± 0.2*
TNF-α	14.1 ± 0.7**
TNF-α + anti-TNF-α	2.1 ± 0.2***
MTV	38.6 ± 6.3†
MTV + TNF-α	61.2 ± 5.6‡
MTV + anti-TNF-α	28.9 ± 6.7§
MTV + TNF-α + anti-TNF-α	29.4 ± 6.5‖
MTV + control antibody	39.1 ± 4.7#

[a] Monocytes were stimulated for 16 h with or without membrane components (MTV, 50 μg/ml) in the presence of TNF-α (20 ng/ml) and/or anti-TNF-α (5 μg/ml). Mouse IgG1 (anti-IL-1β, 5 μg/ml) was used as a control antibody.

[b] Data are expressed as means ± SEs of three separate experiments performed in duplicate. * versus **, $P < 0.001$; ** versus ***, $P < 0.001$; † versus ‡, $P < 0.001$; † versus §, $P < 0.05$; † versus ‖, $P < 0.05$; ‡ versus ‖, $P < 0.001$; § versus #, $P < 0.05$.

mRNA from monocytes treated with LPS was 3.3 ± 0.4 h. However, treatment of monocytes with MTV resulted in an IL-8 mRNA half-life of 1.5 ± 0.2 h ($n = 3$, $P < 0.05$), representing an approximately 55% reduction in the half-life of IL-8 mRNA compared with that observed after LPS stimulation (Fig. 6).

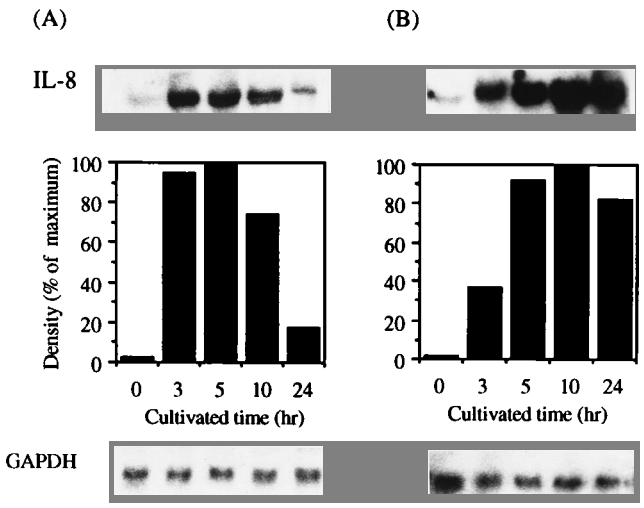


FIG. 4. Time-dependent IL-8 mRNA expression from MTV-treated (A) and LPS-treated (B) monocytes. Upper panels, chemiluminescence exposure of the Northern blot for IL-8 mRNA; middle panels, densitometry of the chemiluminescence exposure; lower panels, chemiluminescence exposure of the Northern blot for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control. Expression of IL-8 mRNA in MTV-treated monocytes reached the maximum at 5 h of cultivation and decreased thereafter.

DISCUSSION

The present study has clearly shown that MTV is a novel inducer responsible for IL-8 generation from monocytes interacting with *T. vaginalis*. Evidence for IL-8 production includes the following: (i) it was identified in a ELISA specific for IL-8; (ii) its chemotactic activity was blocked by a neutralizing monoclonal antibody directed against IL-8 and not an irrelevant antibody; and (iii) IL-8 mRNA expression in MTV-treated monocytes was dose and time dependent.

The nature of components in MTV responsible for the induction of IL-8 by monocytes has not been characterized, but the possible contamination of endotoxin was excluded on the basis of the following evidence: (i) trichomonads were cultured without contamination of bacteria or other microorganisms; (ii) levels of endotoxin in each batch of MTV or culture medium were less than 10 pg/ml, as checked by *Limulus* assay; and (iii) the endotoxin antagonist polymyxin B, an inhibitor of LPS-mediated cytokine production, did not affect MTV-induced IL-8 production. This suggests that the induction of IL-8 production could be ascribed mainly to MTV and not to potential contaminants consisting of minute amounts of endotoxin.

Although *T. vaginalis* and *P. hominis* infect hosts at different anatomical sites, both species have similar antigenic compositions (18). The finding that membrane components of both *T. vaginalis* and *P. hominis* can stimulate monocytes to generate bioactive IL-8 suggests that *T. vaginalis* may have some com-

TABLE 4. Effect of anti-IL-8 monoclonal antibodies on neutrophil chemotaxis induced by MTVCM

Chemoattractant[a]	Neutrophil chemotaxis (no. of cells that migrated) in the presence of[b]:				
	Control[c]	Monoclonal antibodies (5 μg/ml) against:			
		IL-8	GM-CSF	IL-1β	TNF-α
IL-8 (10 nM)	164 ± 17*	2 ± 1**	158 ± 13	155 ± 11	161 ± 15
LTB_4 (10 nM)	112 ± 10	99 ± 5	103 ± 11	104 ± 9	109 ± 9
FMLP (10 nM)	194 ± 11	201 ± 8	199 ± 6	187 ± 17	204 ± 9
CCM	31 ± 6†	19 ± 6	31 ± 4	26 ± 5	22 ± 5
MTVCM	153 ± 6‡	23 ± 3§	154 ± 10	148 ± 12	155 ± 3

[a] The CCM and MTVCM used were taken at 16 h of cultivation.

[b] Data represent means ± SEs of three separate experiments performed in duplicate. * versus **, $P < 0.001$; † versus ‡, $P < 0.001$; ‡ versus §, $P < 0.001$.

[c] Control, in the absence of mouse monoclonal antibodies.

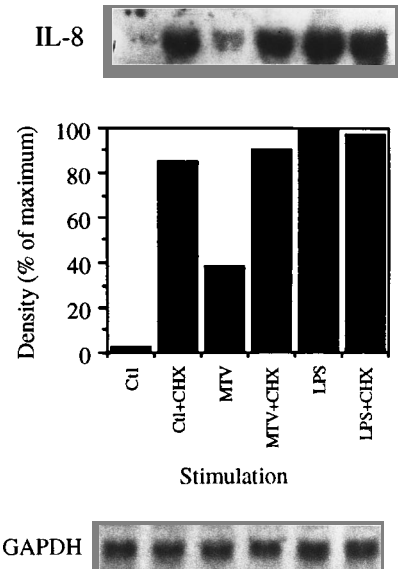


FIG. 5. Effect of cycloheximide (CHX) on the expression of IL-8 mRNA in MTV- and LPS-treated monocytes. Upper panel, chemiluminescence exposure of the Northern blot for IL-8 mRNA; middle panel, densitometry of the chemiluminescence exposure; lower panel, chemiluminescence exposure of the Northern blot for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control. Superinduction of IL-8 mRNA expression was observed when MTV-stimulated monocytes were treated concomitantly with cycloheximide, while no superinduction of IL-8 mRNA expression could be found in LPS-stimulated monocytes.

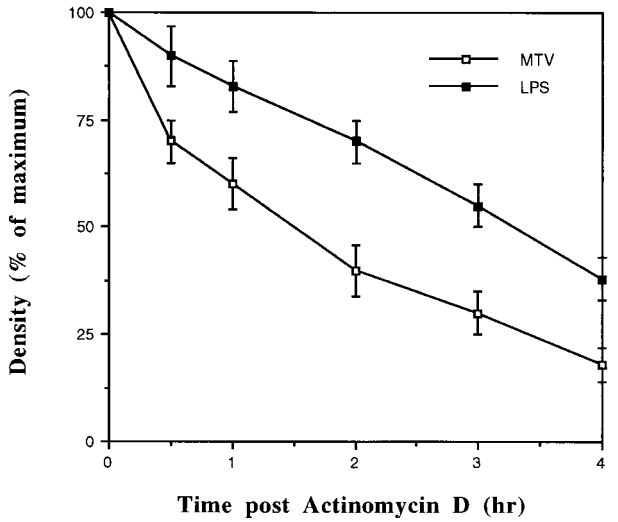


FIG. 6. Effect of MTV and LPS on IL-8 mRNA stability. The half-life of IL-8 mRNA from monocytes treated with LPS was 3.3 ± 0.4 h, while treatment of monocytes with MTV resulted in an IL-8 mRNA half-life of 1.5 ± 0.2 h ($n = 3$), representing an approximately 55% reduction in the half-life compared with that observed after LPS stimulation.

ponents of membrane fractions in common with *P. hominis*, which serve as a novel but not a unique stimulator for IL-8 production by monocytes. Further studies on the nature of the membrane components are being undertaken.

IL-8 is a novel chemotactic cytokine produced by a variety of blood and tissue cells. Epithelial cells from different sources interacting with bacteria have been shown to produce IL-8 (1, 15). In this study, stimulation by *T. vaginalis* or its membrane components, however, failed to demonstrate IL-8 production by human HeLa cervical epithelial cells (data not shown). There is no doubt that adherence to epithelial cells is characteristic of *T. vaginalis* (2). Detachment of epithelial cells by *T. vaginalis* has been well recognized (17). This could result in direct contact of monocytes/macrophages in the submucosa with *T. vaginalis*, with IL-8 produced in response to such an interaction. Our study does not exclude the possible role of neutrophil-derived IL-8 in the inflammatory response elicited by *T. vaginalis* but suggests that monocytes may be the crucial cell source for IL-8 production during *T. vaginalis* infection. On the other hand, the fact that antibody directed against IL-8 suppressed neutrophil chemotaxis induced by MTVCM from 153 to 23 cells but not to 0 (Table 4) indicates that IL-8 contributed to the major chemotactic induction but that some level of non-IL-8-mediated chemotactic activity was present.

It has been shown that phagocytosing or chemotactically stimulated (e.g., by FMLP and LTB_4) neutrophils could express and release large amounts of IL-8 (6, 7, 22). Moreover, the combined presence of IL-8 and GM-CSF at inflammatory foci could result in the synthesis of platelet-activating factor and LTB_4 by neutrophils (23). Therefore, our finding that high levels of IL-8 and LTB_4 were present in vaginal discharges from patients with symptomatic trichomoniasis (31, 34) raises the possibility that neutrophils and other cells capable of producing LTB_4 may induce IL-8 production by inflammatory neutrophils and thereby amplify the acute inflammatory response by recruiting additional neutrophils into an inflammatory site.

Monocytes/macrophages produce IL-8 in response to LPS, TNF-α, or IL-1β (24, 36, 38). It has been shown that both TNF and IL-1 contributed to the subsequent response in biphasic production of IL-8 in LPS-stimulated human whole blood (12). In this study, we found that MTV-induced IL-8 production did not appear to be secondary to IL-1β production since a neutralizing monoclonal anti-IL-1β antibody did not block MTV-induced IL-8 production. On the other hand, the finding that a neutralizing monoclonal anti-TNF-α antibody reduced IL-8 production by MTV-stimulated monocytes suggests that the release of IL-8 in MTVCM is partially dependent on TNF-α. Nevertheless, the observation that MTV can directly induce the expression of IL-8 from human monocytes in the absence of de novo protein synthesis suggests that direct induction of IL-8 in *T. vaginalis* infection is likely to occur.

The superinduction of IL-8 mRNA from unstimulated and MTV-stimulated monocytes by coincubation with cycloheximide is similar to that found in the study of lipoteichoic acid (35). It has been suggested that repressor proteins are produced under resting conditions which inhibit mRNA transcription (11) or accelerate the rate of mRNA decay (20), and these proteins may be inducible in the presence of lipoteichoic acid (35). In contrast, other studies have shown that cycloheximide is unable to superinduce IL-8 mRNA from LPS- or cadmium-stimulated monocytes (19, 35). Furthermore, studies on the mRNA stability revealed that IL-8 mRNA was less stable in the presence of MTV than in the presence of LPS. Taken together, these results suggest that MTV activates the transcription of the IL-8 gene through a pathway or mechanism similar to that activated by lipoteichoic acid rather than that by LPS or cadmium.

The involvement of IL-8 in disease is suggested by the demonstration of its occurrence in the skin lesions of patients with psoriasis (29), by the detection of IL-8 in the bronchoalveolar lavage fluid from patients with adult respiratory distress syndrome (8, 25), and by the substantial quantities of cell-associated IL-8 in specimens from patients with acute staphylococcal endocarditis (35). The findings that IL-8 is present in vaginal discharges from patients with symptomatic trichomoniasis (34) and that the generation of IL-8 in monocytes is stimulated by MTV add to the list of relationships between IL-8 and diseases. IL-8 is rather resistant to inactivation and slow clearance compared with other chemoattractants like FMLP, C5a, LTB_4, and platelet-activating factor (10). The capacity of *T. vaginalis* to induce IL-8 production by monocytes may have in vivo relevance. It is therefore conceivable that IL-8 production, triggered in monocytes directly by trichomonad product or indirectly via release of another cytokine, may play a crucial role in the local accumulation of neutrophils to amplify resistance against *T. vaginalis.*

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REFERENCES

1. **Agace, W., S. Hedges, U. Andersson, J. Andersson, M. Ceska, and C. Svanborg.** 1993. Selective cytokine production by epithelial cells following exposure to *Escherichia coli*. Infect. Immun. **61:**602–609.
2. **Alderete, J. F., M. W. Lehker, and R. Arroyo.** 1995. The mechanisms and molecules involved in cytoadherence and pathogenesis of *Trichomonas vaginalis*. Parasitol. Today **11:**70–74.
3. **Aley, S. B., W. A. Scott, and Z. A. Cohn.** 1980. Plasma membrane of *Entamoeba histolytica*. J. Exp. Med. **152:**391–404.
4. **Al-Sumidaie, A. M., D. L. Jones, and H. L. Young.** 1984. Characterisation of the under-agarose method for quantifying migration of highly purified human monocytes. J. Immunol. Methods **75:**129–140.
5. **Baggiolini, M., A. Walz, and S. L. Kunkel.** 1989. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. J. Clin.

Invest. **84:**1045–1049.
6. **Bazzoni, F., M. A. Cassatella, F. Rossi, M. Ceska, B. Dewald, and M. Baggiolini.** 1991. Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8. J. Exp. Med. **173:**771–774.
7. **Cassatella, M. A., F. Bazzoni, C. Miroslar, I. Ferro, M. Baggiolini, and G. Berton.** 1992. IL-8 production by human polymorphonuclear leukocytes: the chemoattractant formyl-methionyl-leucyl-phenylalanine induces the gene expression and release of IL-8 through a pertussin toxin-sensitive pathway. J. Immunol. **148:**3216–3220.
8. **Chollet-Martin, S., P. Montravers, C. Gibert, C. Elbim, J. M. Desmonts, J. Y. Fagon, and M. A. Gougerot-Pocidalo.** 1993. High levels of interleukin-8 in the blood and alveolar spaces of patients with pneumonia and adult respiratory distress syndrome. Infect. Immun. **61:**4553–4559.
9. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
10. **Colditz, I. G., R. D. Zwahlen, and M. Baggiolini.** 1990. Neutrophil accumulation and plasma leakage induced *in vivo* by neutrophil-activating peptide-1. J. Leukocyte Biol. **48:**129–137.
11. **Collart, M. A., D. Belin, J. Vassalli, S. de Kossodo, and P. Vassalli.** 1986. γ-Interferon enhances macrophage transcription of the tumor necrosis factor/cachectin, interleukin 1, and urokinase genes, which are controlled by short-lived repressors. J. Exp. Med. **164:**2113–2118.
12. **DeForge, L. E., J. S. Kenney, M. L. Jones, J. S. Warren, and D. G. Remick.** 1992. Biphasic production of IL-8 in lipopolysaccharide (LPS)-stimulated human whole blood: separation of LPS-and cytokine-stimulated components using anti-tumor necrosis factor and anti-IL-1 antibodies. J. Immunol. **148:** 2133–2141.
13. **Diamond, L. S., D. R. Harlow, and C. C. Cunnick.** 1978. A new medium for the axenic cultivation of *Entamoeba histolytica* and other entamoeba. Trans. R. Soc. Trop. Med. Hyg. **72:**431–432.
14. **Djeu, J. Y., K. Matsushima, J. J. Oppenheim, K. Shiotsuki, and D. K. Blanchard.** 1990. Functional activation of human neutrophils by recombinant monocyte-derived neutrophil chemotactic factor/IL-8. J. Immunol. **144:** 2205–2210.
15. **Eckmann, L., M. F. Kagnoff, and J. Fierer.** 1993. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. Infect. Immun. **61:** 4569–4574.
16. **Fouts, A. C., and S. J. Kraus.** 1980. *Trichomonas vaginalis*: reevaluation of its clinical presentation and laboratory diagnosis. J. Infect. Dis. **141:**137–143.
17. **Gupta, P. K., and J. K. Frost.** 1990. Cytopathology and histopathology of the female genital tract in *Trichomonas vaginalis* infection, p. 274–290. *In* B. M. Honigberg (ed.), Trichomonads parasitic in humans. Springer-Verlag, New York.
18. **Honigberg, B. M.** 1990. Trichomonads found outside the urogenital tract of humans, p. 342–393. *In* B. M. Honigberg (ed.), Trichomonads parasitic in humans. Springer-Verlag, New York.
19. **Horiguchi, H., N. Mukaida, S. I. Okamoto, H. Teranishi, M. Kasuya, and K. Matsushima.** 1993. Cadmium induces interleukin-8 production in human peripheral blood mononuclear cells with the concomitant generation of superoxide radicals. Lymphokine Cytokine Res. **12:**421–428.
20. **Malter, J. S.** 1987. Identification of an AUUUA-specific messenger RNA binding protein. Science **246:**664–666.
21. **Matsushima, K., K. Morishita, T. Yoshimura, S. Lavu, Y. Kobayashi, W. Lew, E. Appella, H. F. Kung, E. J. Leonard, and J. J. Oppenheim.** 1988. Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin 1 and tumor necrosis factor. J. Exp. Med. **167:**1883–1893.
22. **McCain, R. W., E. P. Holden, T. R. Blackwell, and J. W. Christman.** 1994. Leukotriene B4 stimulates human polymorphonuclear leucocytes to synthesize and release interleukin-8 in vitro. Am. J. Respir. Cell Mol. Biol. **10:**651–657.
23. **McDonald, P. P., M. Pouliot, P. Borgeat, and S. R. McColl.** 1993. Induction by chemokines of lipid mediator synthesis in granulocyte-macrophage colony-stimulating factor-treated human neutrophils. J. Immunol. **151:**6399–6409.
24. **Mukaida, N., and K. Matsushima.** 1992. Regulation of IL-8 production and the characteristics of the receptors for IL-8, p. 41–53. *In* M. Baggiolini and C. Sorg (ed.), Interleukin-8 (NAP-1) and related chemotactic cytokines. Karger, Basel.
25. **Parsons, P. E., A. A. Fowler, T. M. Hyers, and P. M. Henson.** 1985. Chemotactic activity in bronchoalveolar lavage fluid from patients with adult respiratory distress syndrome. Am. Rev. Respir. Dis. **132:**490–493.
26. **Peveri, P., A. Walz, B. Dewald, and M. Baggiolini.** 1988. A novel neutrophil-activating factor produced by human mononuclear phagocytes. J. Exp. Med. **167:**1547–1559.
27. **Rein, M. F., J. A. Sullivan, and G. L. Mandell.** 1980. Trichomonacidal activity of human polymorphonuclear neutrophils: killing by disruption and fragmentation. J. Infect. Dis. **142:**575–585.
28. **Schroder, J. M.** 1989. The monocyte-derived neutrophil activating peptide (NAP/interleukin-8) stimulates human neutrophil arachidonate-5-lipoxygenase, but not the release of cellular arachidonate. J. Exp. Med. **170:**847–863.
29. **Schroder, J. M., and E. Christophers.** 1986. Identification of C5ades arg and an anionic neutrophil-activating peptide (NANP) in psoriatic scales. J. Invest. Dermatol. **87:**53–58.
30. **Shaio, M. F., F. Y. Chang, S. C. Hou, C. S. Lee, and P. R. Lin.** 1991. The role of immunoglobulin and complement in enhancing the respiratory burst of neutrophils against *Trichomonas vaginalis*. Parasite Immunol. **13:**241–250.
31. **Shaio, M. F., and P. R. Lin.** 1995. Leukotriene B4 levels in vaginal discharges from patients with trichomoniasis. Ann. Trop. Med. Parasitol. **89:**85–88.
32. **Shaio, M. F., and P. R. Lin.** 1995. Influence of humoral immunity on leukotriene B4 production by neutrophils in response to *Trichomonas vaginalis* stimulation. Parasite Immunol. **17:**127–133.
33. **Shaio, M. F., P. R. Lin, C. S. Lee, S. C. Hou, P. Tang, and K. D. Yang.** 1992. A novel neutrophil-activating factor released by *Trichomonas vaginalis*. Infect. Immun. **60:**4475–4482.
34. **Shaio, M. F., P. R. Lin, J. Y. Liu, and K. D. Yang.** 1994. Monocyte-derived interleukin-8 involved in the recruitment of neutrophils induced by *Trichomonas vaginalis* infection. J. Infect. Dis. **170:**1638–1640.
35. **Standiford, T. J., D. A. Arenberg, J. M. Danforth, S. L. Kunkel, G. M. VanOtteren, and R. M. Strieter.** 1994. Lipoteichoic acid induces secretion of interleukin-8 from human blood monocytes: a cellular and molecular analysis. Infect. Immun. **62:**119–125.
36. **Standiford, T. J., R. M. Strieter, S. W. Chensue, J. Westwick, K. Kasahara, and S. L. Kunkel.** 1990. IL-4 inhibits the expression of IL-8 from stimulated human monocytes. J. Immunol. **145:**1435–1439.
37. **Stone, R. M., K. Imamura, R. Datta, M. L. Sherman, and D. W. Kufe.** 1990. Inhibition of phorbol ester-induced monocytic differentiation and c-fms gene expression by dexamethasone: potential involvement of arachidonic acid metabolites. Blood **76:**1225–1232.
38. **Strieter, R. M., S. W. Chensue, M. A. Basha, T. J. Standiford, J. P. Lynch III, M. Baggiolini, and S. L. Kunkel.** 1990. Human alveolar macrophage gene expression of interleukin-8 by TNF-α and IL-1β. Am. J. Respir. Cell Mol. Biol. **2:**321–326.
39. **Tso, J. Y., X. H. Sun, T. H. Kao, K. S. Reece, and R. Wu.** 1985. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. Nucleic Acids Res. **13:**2485–2502.